Biologically Active Glycosides from Asteroidea, 39[+] Glycosphingolipids from the Starfish *Linckia laevigata*, 1

Isolation and Structure of a New Ganglioside Molecular Species

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A ganglioside molecular species [LLG-3 (**1**)] has been obtained from the water-soluble lipid fraction of the $CHCl_3$/MeOH extract of the starfish *Linckia laevigata*. On the basis of chemical and spectroscopic findings, the structure of **1** has been elucidated. Negative-ion FABMS provided important information concerning both the structure of the sugar moiety and the molecular mass of the ganglioside. On the other hand, positive-ion FABMS/MS of $[M + Na]^+$ ions obtained in the positive-ion FABMS of the ceramide lactoside (**4**) derived from **1** indicated the structure of the fatty acid chain of the ceramide moiety. **1** represents a new ganglioside molecular species possessing a 2→11-linked tandem-type disialosyl moiety.

In our ongoing search for biologically active glycosphingolipids from starfish, we have isolated numerous cerebrosides, ceramide-lactosides, sulfatides, and gangliosides, and some of them have biological activities.[1] In continuation of the previous studies, we have now carried out the isolation and structure elucidation of the biologically active glycosphingolipids from the starfish *Linckia laevigata* (Aohitode in Japanese), with the objective of searching for lead compounds for new medicines. We describe here the isolation and structure determination of a new ganglioside from the whole bodies of *L. laevigata*.

A water-soluble lipid fraction, obtained from the $CHCl_3$/MeOH extract of the whole bodies of *L. laevigata*, was subjected to reversed-phase followed by normal-phase column chromatography, and then to Sephadex LH-20 column chromatography to give a ganglioside molecular species, LLG-3 (**1**). The isolated material gave a single spot upon normal-phase thin-layer chromatography (TLC).

In its IR spectrum, **1** exhibits strong hydroxy (3400 cm^{-1}) and amide (1650, 1560 cm^{-1}) absorptions, while the ^{13}C-NMR spectrum features the characteristic signals of a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acid function and a sugar moiety at C-1 (Table 1) [δ = 70.3 (C-1), 51.6 (C-2), 75.6 (C-3), 72.7 (C-4), 176.2 (C-1′) and 72.7 (C-2′)]. Furthermore, **1** is thought to possess a *normal*[2] type of side chain, mainly, since the carbon-atom signals due to the terminal methyl groups were observed at δ = 14.5 in the ^{13}C-NMR spectrum. The signals due to a small amount of an *ante-iso*[2] type of terminal methyl groups are observed at δ = 11.8 (terminal methyl group) and 19.6 (branched methyl group).

The ^{13}C-NMR spectrum of **1** also features a signal due to a methoxy carbon atom at δ = 58.6, along with those of four anomeric carbon atoms at δ = 100.2, 101.3, 104.9, 105.2, two of which (δ = 100.2, 101.3) are quaternary carbon signals and thus indicate the presence of two sialic acid residues. The negative-ion fast-atom bombardment mass spectrum (FABMS) exhibits a series of molecular ion peaks due to anionized cluster ions $[M - H]^-$ at *m/z* = 1576, 1590, and 1604. Therefore, **1** is suggested to be a molecular species of a phytosphingosine-type ganglioside possessing a 2-hydroxy fatty acid moiety, a methoxy group, and four monosaccharide units. The fatty acid and long-chain base (phytosphingosine) constituents, as well as the structure of the oligosaccharide moiety of **1**, were determined as follows.

The structure of the ceramide moiety was investigated first. **1** was hydrolyzed with 5% aq. AcOH to give ceramide dihexoside (**4**, Scheme 1). In the positive-ion FABMS of **4**, a series of $[M + Na]^+$ ions of molecular species was observed in the range *m/z* = 970−1050, as shown in Scheme 2a. When positive-ion FABMS/MS of each of the $[M + Na]^+$ ions were measured, prominent fragment ions originating from cleavage of the amide bond, for example at *m/z* = 650 from the $[M + Na]^+$ ion with *m/z* = 1002, were observed (Scheme 2b).[3] On the basis of these $[M + Na]^+$ ions and the fragment ions derived therefrom, we could deduce the fatty acid and long-chain base components of each molecular species in **4**, as shown in Table 2.

The stereochemistry of the ceramide moiety was determined as follows. The ^1H- and ^{13}C-NMR spectra of compound **4** closely resemble those of the synthetic lactosyl ceramide, (2*S*,3*S*,4*R*)-1-*O*-[*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-2-[(2*R*)-2-hydroxytetracosanoylamino]-

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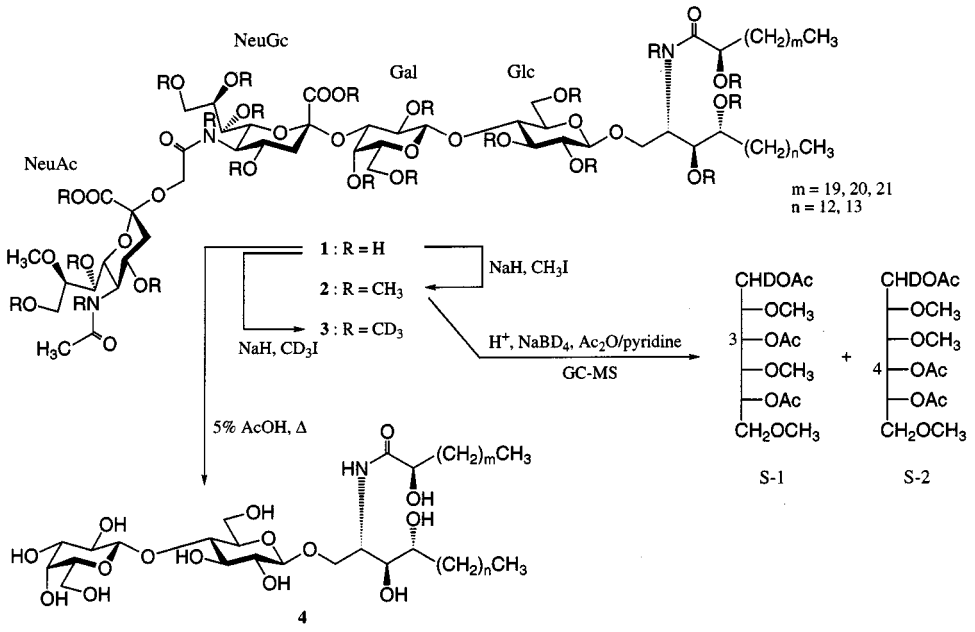


Scheme 1

Table 1. ^{13}C-NMR spectral data of LLG-3(**1**), LLG-3 CDH(**4**) and synthetic lactosyl ceramide(**5**) [δ values in C_5D_5N/D_2O (95:5) (**1**), C_5D_5N (**4**, **5**)]

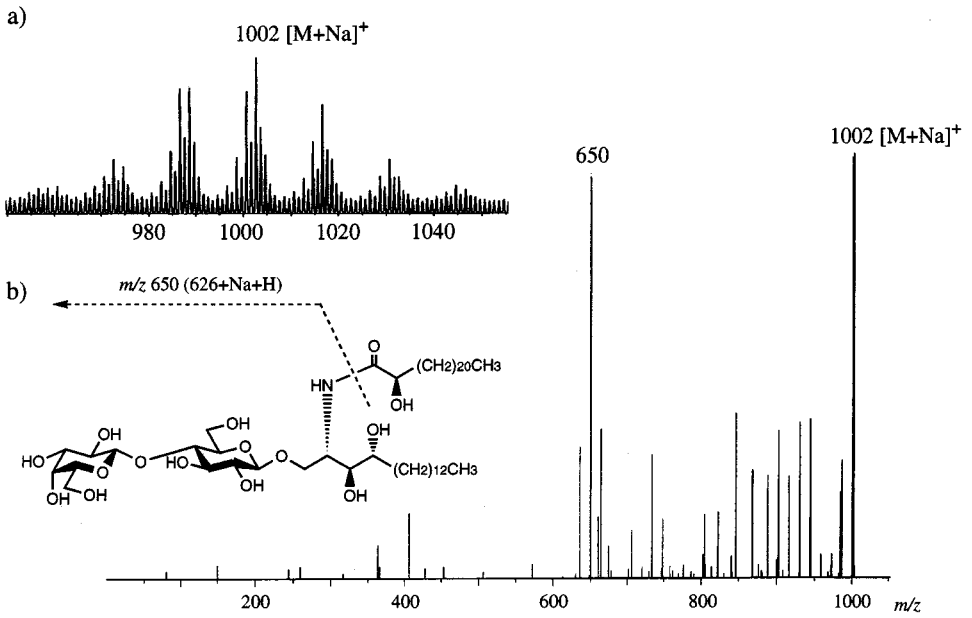
C			1	4	5[a]
ceramide	1	(t)	70.3	70.2	70.3
	2	(d)	51.6	51.6	51.6
	3	(d)	75.6	75.8	75.8
	4	(d)	72.7	72.4 *	72.6
	1'	(s)	176.2	175.5	175.6
	2'	(d)	72.7	72.5 *	72.6
-CH_3		(q)	14.5	14.3	14.3
Glc	1	(d)	104.9	105.0	105.0
	2	(d)	74.5	74.6	74.6
	3	(d)	76.4	76.5	76.5
	4	(d)	81.5	81.6	81.7
	5	(d)	77.1	77.2	77.2
	6	(t)	62.0 *	62.0	62.1 *
Gal	1	(d)	105.2	105.6	105.8
	2	(d)	72.7	72.3	72.4
	3	(d)	81.9	75.1	75.2
	4	(d)	70.3	70.0	70.1
	5	(d)	76.4	76.5	76.5
	6	(t)	62.1 *	62.0	62.0 *
NeuGc	1	(s)	173.9 **		
	2	(s)	100.2 ***		
	3	(t)	42.7		
-NHCOCH$_2$O-		(s)	174.3 **		
NeuAc	1	(s)	173.9 **		
	2	(s)	101.3 ***		
	3	(t)	42.7		
-NHCOCH$_3$		(s)	174.3 **		
-OCH_3		(q)	58.6		

*,**,***: Assignments may be interchanged in each vertical column. – [a]Data from ref.[4]

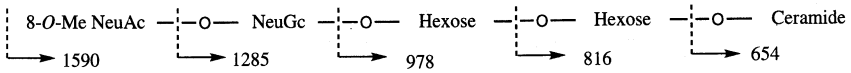
1,3,4-hexadecanetriol (**5**)[4] (Table 1). This fact, together with the similar optical rotations of **4** (+12.9) and **5** (+8.0), indicates that **4** must be a ceramide lactoside having the same absolute configuration of the core structure as **5** (C-2, C-3, C-4, C-2′, and lactose). Therefore, the absolute configuration of the ceramide part of the parent ganglioside **1** must be 2*S*,3*S*,4*R*,2′*R* (Scheme 1).

Next, the structure of the sugar moiety of **1** was examined. In the negative-ion FABMS, the molecular ion and fragment ion peaks arising from cleavage of the glycoside linkages of the major component are observed at *m*/*z* = 1590, 1285, 978, 816, and 654 (Scheme 3). Since the presence of a lactosyl ceramide moiety had already been established, the linear carbohydrate sequence of **1** could be deduced as being *O*-Me-NeuAc→NeuGc→β-Gal*p*-(1→4)-β-Glc*p*. Methylation of **1** according to the Hakomori method[5] afforded the permethylated product **2**. Partially methylated alditol acetates prepared from **2** were analyzed by GC MS and identified as the alditols derived from 3-linked hexopyranose (S-1) and 4-linked hexopyranose (S-2) (Scheme 1). The structure of the sialic acid moiety was established as follows. Since **1** has a methoxy group on its terminal sialic acid residue, pertrideuteriomethylated product **3** was prepared, then methanolyzed, and finally acetylated. The acetylated partially trideuteriomethylated sialic acid derivatives (S-3 and S-4) were then examined by means of GC MS and gave characteristic fragment ion peaks (Scheme 4) that indicated the presence of terminal 8-*O*-Me-NeuAc and 11-linked NeuGc in a ratio of ca. 1:1.

On the basis of the above evidence, the tetrasaccharide moiety of **1** must be 8-*O*-Me-NeuAc-(2→11)-NeuGc-(2→3)-β-Gal*p*-(14)-β-Glc*p*. The configurations of the sialic acid residues (NeuAc and NeuGc) are presumed to be α since their anomeric carbon signals are in good agreement with those of known gangliosides possessing α-linked sialic acids.[6]



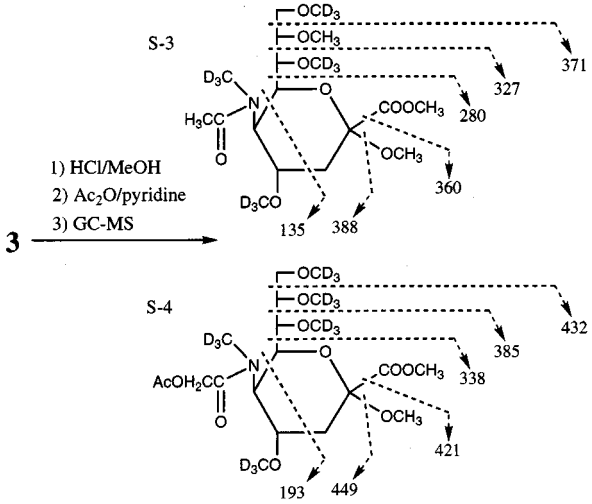
Scheme 2. a) Positive-ion FABMS of **4**; b) positive-ion FABMS/MS of $[M + Na]^+$ ion obtained in the positive-ion FABMS of **4**



Scheme 3. Negative-ion FABMS fragmentation of the major component of **1**

Table 2. Fatty acid and long-chain base component of molecular species in **4**

$[M+Na]^+$	Fatty Acid	Long-Chain-Base
986	α-OH docosanoic acid ($C_{22:0}$)	$C_{17:1}$ -phytosphingosine
988	α-OH docosanoic acid ($C_{22:0}$)	$C_{17:0}$ -phytosphingosine
1000	α-OH tricosanoic acid ($C_{23:0}$)	$C_{17:1}$ -phytosphingosine
1002	α-OH tricosanoic acid ($C_{23:0}$)	$C_{17:0}$ -phytosphingosine
1016	α-OH tricosanoic acid ($C_{23:0}$)	$C_{18:0}$ -phytosphingosine



Scheme 4. EIMS fragmentation of S-3 and S-4

Consequently, if Glc, Gal, NeuAc, and NeuGc are assumed to belong to the most commonly found D series, then LLG-3 (**1**) is the *O*-8-*O*-methyl (*N*-acetyl-α-D-neuraminosyl)-(2→11)-*O*-(*N*-glycolyl-α-D-neuraminosyl)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranoside of a ceramide composed of heterogeneous phytosphingosine and 2-hydroxy fatty acid units. The major components of the fatty acid and long-chain base moieties of **1** are (2*R*)-2-hydroxytricosanoic acid and (2*S*,3*S*,4*R*)-2-amino-1,3,4-heptadecanetriol (Scheme 1).

To the best of our knowledge, this is the first time that a ganglioside has been isolated and characterized from the starfish *L. laevigata*. Furthermore, compound **1** represents a new ganglioside molecular species, and is only the second starfish ganglioside found to contain a 2→11-linked tandem-type disialosyl moiety, after a ganglioside from *Aphelasterias japonica*.[7] The biological activities of **1** will be examined in due course.

Experimental Section

Melting points: Micro melting point apparatus (Yanaco MP-3); uncorrected values. – IR spectra: Jasco IR-700 infrared spectrophotometer. – Optical rotations: Jasco Dip-370 digital polarimeter at 28 °C. – NMR spectra: Instrumentation and techniques were as described in the previous paper,[6] except that the ^{13}C-NMR spec-

trum was measured with a Jeol GX-270 spectrometer. − FAB MS and FAB MS/MS: Jeol JMS-SX/SX102A four-sector-type tandem mass spectrometer of *BE*/*BE* geometry [xenon atom beam: 5 kV, ion-source accelerating potential: 10 kV, matrix: HMPA/TEG (negative-ion mode), *m*NBA/NaCl (positive-ion mode)]. The $[M + Na]^+$ ions were selected as precursor ions and were then subjected to high-energy (10 kV) collision with argon molecules in the third field-free region. The argon pressure was sufficient to attenuate the primary ion beam by 50%. The fragment ions were dispersed by a second spectrometer and CID spectra were recorded. − GC MS: Shimadzu QP-1000 [EI mode; ionization potential: 70 eV; separator and ion-source temperature: 250°C; column: Shimadzu CBP-10-W12-100 (0.53 mm × 12 m); carrier gas: He (30 mL/min)].

Separation of LLG-3 (1): Whole bodies of the starfish *Linckia laevigata* (wet weight 18 kg), collected at Okinawa, Japan, in May 1995, were chopped and extracted with $CHCl_3$/MeOH (1:3, 15 L) and then further extracted with $CHCl_3$/MeOH (1:2, 2 × 12 L). The combined extracts were concentrated in vacuo to give a condensed extract (1 L). This was added to H_2O (1 L) and the resulting mixture was extracted with AcOEt/*n*BuOH (2:1, 3 × 1 L) in order to separate less polar lipids. The aqueous layer was further extracted with *n*BuOH saturated with H_2O (3 × 500 mL) to remove saponins, and the remaining aqueous phase was concentrated in vacuo to give a brown syrup (470 g). This syrup was added to 60% MeOH (2 L) and chromatographed on Cosmosil 140C_{18}-PREP (reversed-phase) [solvent 60%, 80%, 100% MeOH, and $CHCl_3$/MeOH (3:7)] to give four fractions. The crude glycosphingolipid fraction (100% MeOH and $CHCl_3$/MeOH eluate, 3.9 g) was further chromatographed on silica gel [$CHCl_3$/MeOH/H_2O (6:4:0.5 → 6:4:0.7 → 6:4:1)] and then on Sephadex LH-20 [$CHCl_3$/MeOH/H_2O (6:4:1)] to afford **1** (58 mg). **1** showed a single spot upon TLC on silica gel [solvent $CHCl_3$/MeOH/H_2O (6:4:1)], R_f = 0.42.

LLG-3 (1): Amorphous powder; m.p. 245−250°C. − IR (KBr): $\tilde{\nu}$ = 3400 cm^{-1} (OH), 1650, 1560 (amide). − Negative-ion FABMS: *m*/*z* = 1576, 1590, 1604 $[M - H]^-$, 1285, 978, 816, 654 (fragment ions of major component, see Scheme 4). − ^{13}C NMR: See Table 1.

Partial Hydrolysis of 1: Compound **1** (15 mg) was heated with 5% aq. AcOH (8 mL) at 90°C for 4 h. The mixture was then extracted with AcOEt/*n*BuOH (3:1), the organic layer was concentrated in vacuo, and the residue was chromatographed on silica gel [$CHCl_3$/MeOH/H_2O (8:2:0.2)] to give **4** (3.5 mg); $[\alpha]_D$ = +12.9 (*c* = 0.32 in $CHCl_3$/MeOH, 1:1). Compound **4** was identified as a ceramide-lactoside by comparison [^1H- and ^{13}C-NMR data (Table 1)] with the synthetic ceramide-lactoside **5** {ref.[4]: $[\alpha]_D$ = +8.0 (*c* = 0.2 in $CHCl_3$/MeOH, 1:1)}.

Methylation (Trideuteriomethylation) of 1 (Hakomori Method): Compound **1** (ca. 5 mg) was treated with NaH (20 mg) and CH_3I (CD_3I, 0.1 mL) in DMSO (1 mL) according to the Hakomori method. The reaction mixture was subsequently diluted with H_2O and extracted with $CHCl_3$. The combined $CHCl_3$ extracts were washed with H_2O, dried with Na_2SO_4, and the solvent was evaporated in vacuo. The residue was chromatographed on silica gel [$CHCl_3$/acetone (2:1)] to give **2** (**3**) (ca. 1 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 2: Compound **2** (1 mg) was heated with 90% HCOOH/10% CF_3COOH (1:1, 1 mL) at 90°C for 22 h in a sealed small-volume vial. The mixture was then concentrated in vacuo, the residue was dissolved in H_2O (2 mL), and the resulting solution was treated with 28% aq. NH_3 (two drops) and $NaBD_4$ (10 mg). After leaving the mixture to stand at room temp. for a few hours, it was acidified with AcOH to pH = 3.5 and then concentrated in vacuo. H_3BO_3 present in the residue was removed by co-distillation with MeOH (four times). The residue was then heated with Ac_2O/C_5H_5N (1:1, 0.5 mL) at 70°C for 30 min. After concentration of the mixture in vacuo, the residue obtained was extracted with $CHCl_3$, and the combined $CHCl_3$ extracts were washed with H_2O and dried (Na_2SO_4). Evaporation of the solvent left the partially methylated alditol acetates. These acetates were subjected to GC MS [column temp.: 150°C (constant)]. The results were as follows: t_R [min] = 18.5, *m*/*z*: 45, 118, 161, 234 [1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (S-1, derived from 3-linked hexopyranose)]; t_R [min] = 19.5, *m*/*z*: 45, 118, 233 [1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (S-2, derived from 4-linked hexopyranose)].

Preparation and GC-MS Analysis of Partially Trideuteriomethylated Neuraminic Acid Derivatives from 3: Compound **3** (1 mg) was heated with 5% HCl/MeOH (1 mL) at 70°C for 4 h in a sealed small-volume vial. The reaction mixture was then concentrated in vacuo, and the residue (methanolyzate) was heated with Ac_2O/C_5H_5N (1:1, 1 mL) at 70°C for 2 h. The resulting mixture was then concentrated in vacuo, and the residue was subjected to GC MS [column temp.: 180−250°C (rate of temp. increase 4°C/min)]: t_R [min] = 4.2, *m*/*z*: 135, 260, 280, 301, 327, 360, 371, 388 [methyl *N*-acetyl-*N*-trideuteriomethyl-2,8-di-*O*-methyl-4,7,9-tri-*O*-trideuteriomethylneuraminate (S-3, from terminal 8-*O*-MeNeuAc)]; t_R [min] = 8.1, *m*/*z*: 193, 318, 338, 362, 385, 421, 449 [methyl *N*-glycolyl-11-*O*-acetyl-*N*-trideuteriomethyl-2-*O*-methyl-4,7,8,9-tetra-*O*-trideuteriomethylneuraminate (S-4, from 11-linked NeuGc)].

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[2] *Normal* means straight chain (−$CH_2CH_2CH_2CH_3$), *ante-iso* means branched chain possessing a methyl group on the third carbon atom of the terminal methyl group [−$CH_2CH(CH_3)CH_2CH_3$].
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